DEVELOPMENT OF SECONDARY METABOLITES CONJUGATED WITH NANO POLYMERS AGAINST POLY MICROBIAL INFECTIONS

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
IN
MICROBIOLOGY

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THIS CERTIFICATE IS PROUDLY PRESENTED TO

Anurati

Mandal

Who has completed a 90-days internship in DEVELOPMENT OF SECONDARY METABOLITES CONJUGATED WITH NANO POLYMERS AGAINST POLY MICROBIAL INFECTIONS IN CULTURE from 12th February 2025 to 20th May 2025 at our premises. We proudly present this certificate for your hard work, dedication, sincerity, perseverance and punctuality. We wish you all the best in your future endeavours.

20/05/2025
DATE OF ISSUE

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I hereby declare that the work which is being presented in this dissertation entitled "DEVELOPMENT OF SECONDARY METABOLITES CONJUGATED WITH NANO POLYMERS AGAINST POLY MICROBIAL INFECTIONS IN CULTUR" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Microbiology is an authentic record of my own work carried out during the period from February 2025 to May 2025 under the supervision of Mr Mayuk Bose Department of Biotechnology. I acknowledge that I have read and understood the UGC regulations, 2018 (*Promotion of Academic integrity and Prevention of Plagiarism in Higher Educational Institutions*).

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Anurati Mandal
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(Signature of the students with Date)

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TO WHOM IT MAY CONCERN

This is to certify that the project report titled "DEVELOPMENT OF SECONDARY METABOLITES CONJUGATED WITH NANO POLYMERS AGAINST POLY MICROBIAL INFECTIONS", submitted by Ms. Anurati Mandal (Registration No:230010161830 Enrolment No:2322211001016) to the Department of Microbiology/Biotechnology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by her for the partial fulfillment of the requirements for the Master of Science degree.

Date: 06/06/2025
Place: Newtown ,Kolkata

(Signature of Head of the Department)

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ABBREVIATIONS

- **TLC: Thin Layer Chromatography**
- **TPP: Sodium tripolyphosphate**
- **MIC: Minimum inhibitory concentration**
- **ZOI: Zone Of Inhibition**
- **LB Agar: Luria Bertani Agar**
- **PDA: Potato Dextrose Agar**

ABSTRACT

Limonene, a naturally occurring monocyclic monoterpene, is likely one of the principle constituents of citronaceous osnovoy oils and possesses remarkable industrial importance due to its use in food flavoring, cosmetics, medicine, and biopertroleum solvents. This research work focuses on the retrieval, purification and partitioning of limonene from sweet lime (Citrus limetta) waste peels which are normally regarded as waste. The main aim is to develop a recovery technique that is environment-friendly and economically viable and enhances the value of agricultural wastes.After Extraction, Isolation and Purification of the sample ,we did nano particle preparation of chitosan.Then we nano coated our sample. although it is a oil sample so directly it is not mixed with chitosan.So first we take 5.4ml limonene sample then mixed with 20ml distilled water.Then,let the solution on magnetic sterile for 1 hour.Later 40ml HCL added drop wise in the solution. Now the chitosan added 15ml dropwise in the solution.Then heated the sample for 15 minutes at 50 degree C.Now,Filtered the sample to let the oil to float on the surface.Let the solution be on magnetic stirrer for 1hr.Now we have to maintain the ph of the solution .Ideal pH is 4.8.So,first after measuring the pH of the solution ,it came around pH=1.For increasing the PH we used NaOH pallet . after give 6 NaOh pallet the pH is getting increase and get the pH 4.8.By converting the solution into salt we get the ideal pH. Then we performed MIC of 2 poly microbial sets and done IC50 with the same polymicrobial set.And fungal MIC and IC50 we did using a strain of fungi. Then, we did ZOI both bacteria and fungi.After carried out all of the experiments from the extraction of limonene from sweet lime,we can easily conclude that the extracted limonene has antimicrobial property.

KEYWORDS

Limonene , Sweetlime, Enterococcus faecalish,
Listeria monocytogenes, TLC, Zone of inhibition

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INTRODUCTION

Limonene, found in citrus oils, such as lemons and oranges, has a monocyclic structure with a chemical makeup of $C_{10}H_{16}$. Citrus fruits contain rinds that predominate its existence in the form of D-isomer, which gives lemongrass its distinctive flavor. It is being actively pursued in the industry and various scientific fields owing to its economic significance and versatility in agriculture, pharmaceuticals, green chemistry, and flavor industries. Also, because of its anti-inflammatory and potential anti-cancer properties, limonene is being explored as a therapeutic agent, diabolic cleanser, natural solvent of greener origins and an environmentally-friendly cleaner.Eco-friendly extraction practices include purification, extraction, and the processes of limonene isolation. The triadic synergetic system forms the provincial axis of accomplishing optim maximization of the results. Coping with myriade variants and multileveled tasks entails to layer the extracted system using a special space approach while systematically remaining in the bounds of provisional means.Enhanced technology deals with multiplicity of selection of streams like refined air scrubbing, concentrated watery solution, steam distillation, and solvent distillation. Instead of harmful organic solvents which need to be disposed of, separating the compound via steam distillation becomes very beneficial and convenient for laboratories and industries because of its uncomplicated approach, ultimate effectiveness and commonly known steam sifting methods and practice.Limonene exerts marked inhibitory activity against different pathogens, such as with *Listeria monocytogenes*, a bacterium that challenges food safety. It binds to the cell membrane and interrupts its structure, which causes the membrane to become permeable and leakage of its cytosol. Furthermore, limonene blocks bacterial energy metabolism by disrupting the respiratory chain and ATPase, which disables the synthesis of ATP. In addition, it has been documented that limonene can lessen the severity of *Pseudomonas aeruginosa* infection by blocking quorum sensing. *The Pseudomonas aeruginosa* biofilm accounts for 80% of the total biofilm formed. This leads to decreased biofilm development and the bioinduced virulence factors elastase and pyocyanin.Also, limonene proved to exhibit vigorous antifungal activity. *Trichophyton rubrum*, one of the dermatophytes that cause onychomycosis, was inhibited in vitro. At a concentration of 0.5% v/v, limonene demonstrates this effect with C at this concentration.

REVIEW OF LITERATURE

Limonene, a naturally occurring monocyclic monoterpene, is likely one of the principle constituents of citronaceous osnovoy oils and possesses remarkable industrial importance due to its use in food flavoring, cosmetics, medicine, and biopertroleum solvents. This research work focuses on the retrieval, purification and partitioning of limonene from sweet lime (Citrus limetta) waste peels which are normally regarded as waste. The main aim is to develop a recovery technique that is environment-friendly and economically viable and enhances the value of agricultural wastes.Limonene, found in citrus oils, such as lemons and oranges, has a monocyclic structure with a chemical makeup of $C_{10}H_{16}$. Citrus fruits contain rinds that predominate its existence in the form of D-isomer, which gives lemongrass its distinctive flavor. It is being actively pursued in the industry and various scientific fields owing to its economic significance and versatility in agriculture, pharmaceuticals, green chemistry, and flavor industries. Also, because of its anti-inflammatory and potential anti-cancer properties, limonene is being explored as a therapeutic agent, diabolic cleanser, natural solvent of greener origins and an environmentally-friendly cleaner.Eco-friendly extraction practices include purification, extraction, and the processes of limonene isolation. Enhanced technology deals with multiplicity of selection of streams like refined air scrubbing, concentrated watery solution, steam distillation, and solvent distillation. Instead of harmful organic solvents which need to be disposed of, separating the compound via steam distillation becomes very beneficial and convenient for laboratories and industries because of its uncomplicated approach, ultimate effectiveness and commonly known steam sifting methods and practice.Limonene exerts marked inhibitory activity against different pathogens, such as with *Listeria monocytogenes*, a bacterium that challenges food safety. It binds to the cell membrane and interrupts its structure, which causes the membrane to become permeable and leakage of its cytosol. Furthermore, limonene blocks bacterial energy metabolism by disrupting the respiratory chain and ATPase, which disables the synthesis of ATP. In addition, it has been documented that limonene can lessen the severity of *Pseudomonas aeruginosa* infection by blocking quorum sensing. *The Pseudomonas aeruginosa* biofilm accounts for 80% of the total biofilm formed. This leads to decreased biofilm development and the bioinduced virulence factors elastase and pyocyanin.Also, limonene proved to exhibit vigorous antifungal activity. *Trichophyton rubrum*, one of the dermatophytes that cause onychomycosis, was

inhibited in vitro. At a concentration of 0.5% v/v, limonene demonstrates this effect with C at this concentration. As well, it limits the growth and virulence of highly pathogenic yeast known as *Candida parapsilosis* by blocking sculpting.

MATERIALS AND METHODS

- **Extraction, Isolation and Purification of Secondary Metabolites:** Graded sweet lime peels from approximately 23 sweet lime are taken.

I. Extraction

1.The graded peels are transferred to a round bottom flask.
2.To the peels, 400 ml of distilled water is poured.
3.A simple water bath distillation is carried out.
4.Two separate regions will form in the distillate portion, the upper portion making limonene.

Fig1: Graded sweet lime peels

Isolation and purification

The whole distillate is transferred to a separatory funnel to separate out the upper limonene layer.

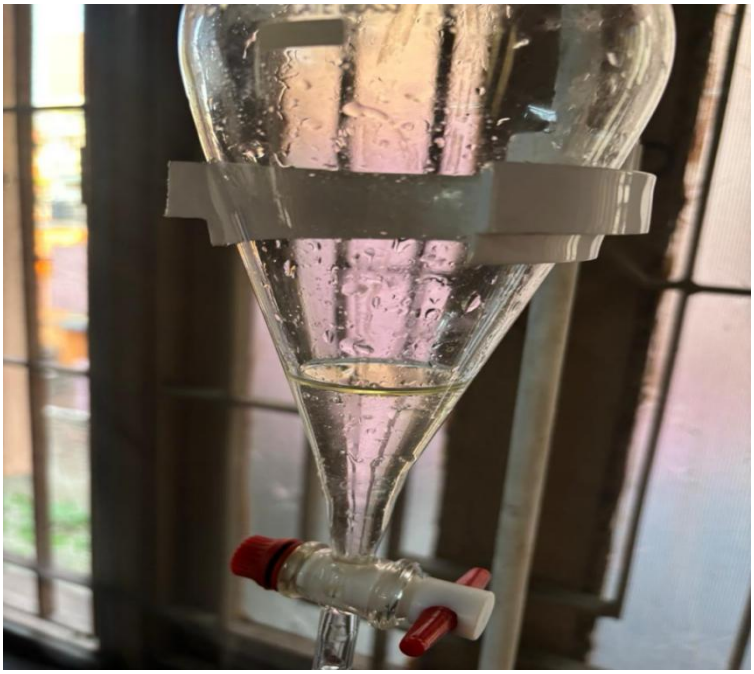

Fig2: Used separation funnel to separate oil and water

❖ Biochemical test

2ml of chloroform is taken and mixed with 0.5 ml of sample. Conc H2SO4 added drop wise. Then, ring of red brown colour forms monoterpenoid present and confirmed.

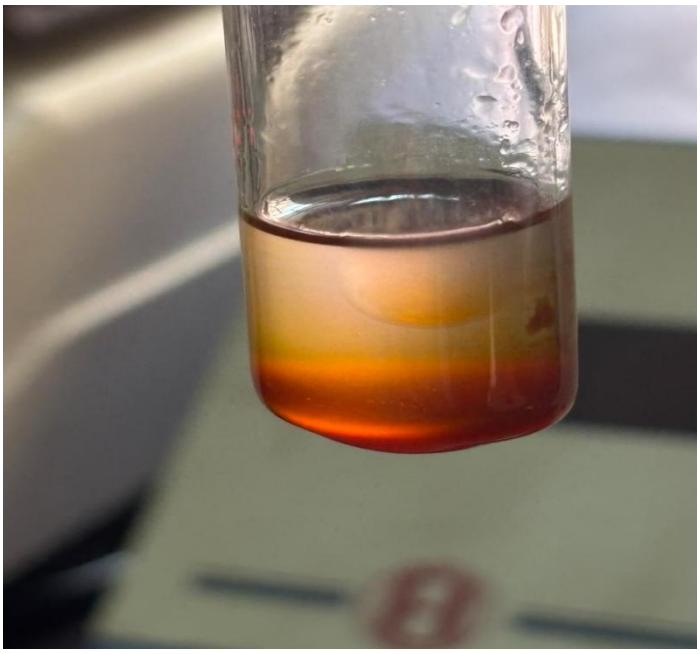

Fig3: Red brown colour ring forms means monoterpenoid present and confirmed.

❖ TLC (Thin Layer Chromatography)

Silica is powdered using mortar pestle and distilled water is added then dried into the air oven forming a thin layer. In TLC ,Rf value or Retention Factor explain the distance between the sample and the solvent. So Rf factor is basically defined as the distance travelled by the sample divided by distance travelled by the solvent.
For TLC Chloroform 16ml and Hexon 4ml mixed like 8:2 ratio.

Rf value =Sample distance/solvent distance
1.7/5.4 = .315

The RF value given in protocol is .2 to.3
And,the RF I got is .3.So the purity is maintained by doing TLC.

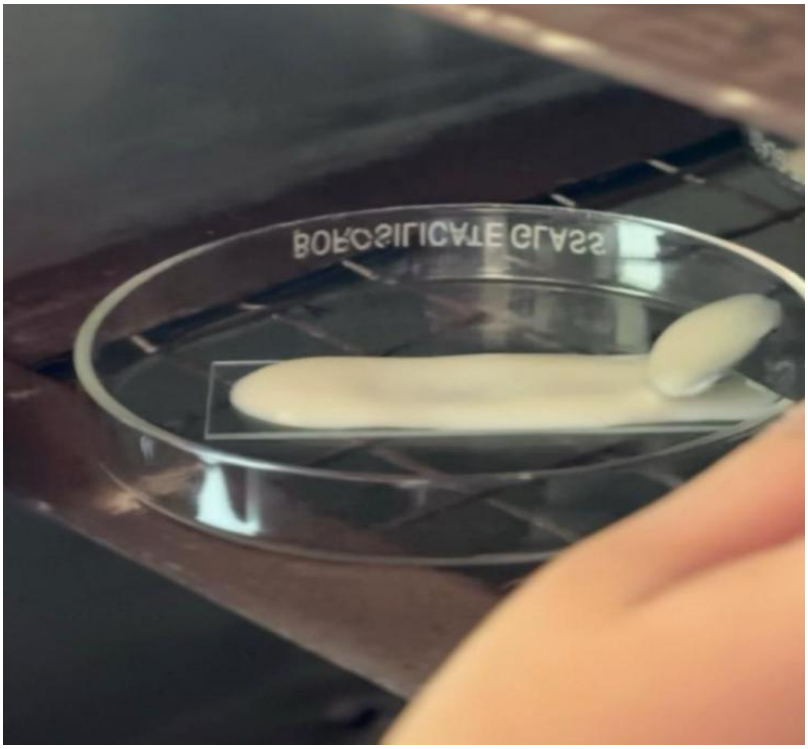

Fig4: Thin layer of silica

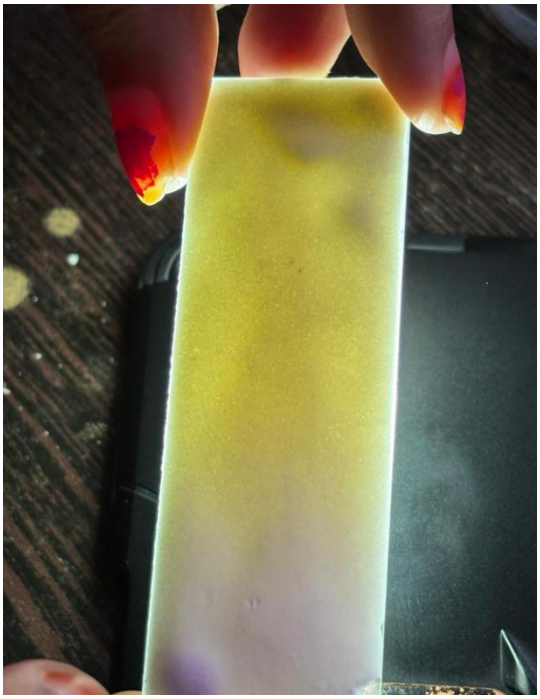

Fig5: Result of thin layer chromatography

❖ UV spectrophotometer analysis

Maintain the OD at 237 nm, after that the uv spectra result is 0.023
this result is same that given in the protocol.

❖ Nano particle preparation of chitosan

- 50 mg of chitosan powder dissolved in 50mL deionized distilled water.
- After in at room temperature, the solution placed on the magnetic stirring and 1% acetic acid solution added dropwise within 30 mins to create the homogeneous. chitosan solution.
- Sodium tripolyphosphate [TPP 0.5% (W/V), 2mL] as a crosslinking agent added drop wise and the compound will be stirred 30 meus.
- 2.0 mg/mL ICG added to the prepared chitosan solution and mixed via a magnetic mixer for 10 meus at 1000 rpm, în at pH 4.8.

- Eventually, this solution centrifuged at 10,000 ppm for 30mins.
- The entire chitosan nanoparticle solution is passed through a 0.22 micron syringe filter to make sure particles below the range of 200 nm passes through the solution after which the entire solution is scanned from 200-800 nm in spectrophotometer which should give a peak concluding nanoparticles are formed.

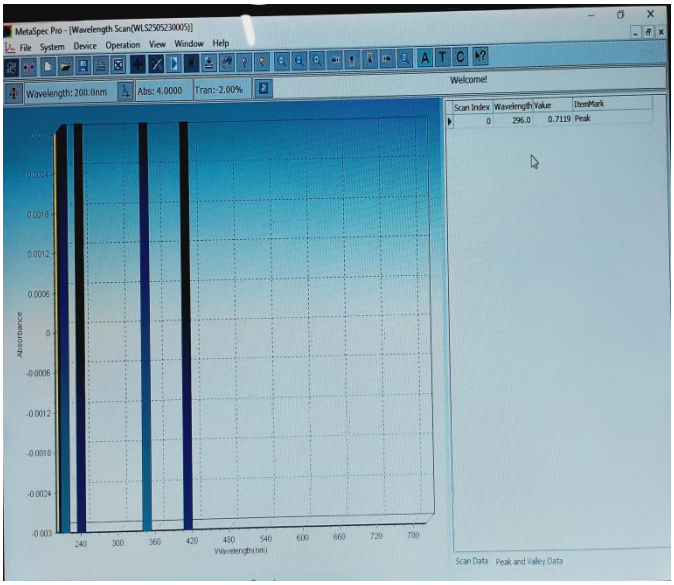

Fig6: The UV range in spectrophotometer without the coating of the drug

Fig7: Filtration of Chitosan nanoparticles

- Once the nanoparticles are coated with drugs again the solution is scanned through a UV Spectrophotometer from 200-800 nm and again the peak is observed.

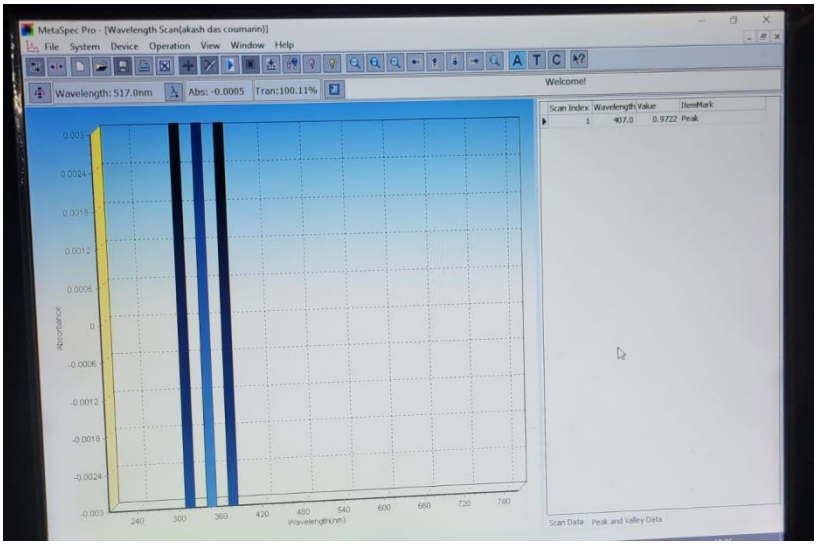

Fig8: The UV range in spectrophotometer after the coating of the drug

Now in comparision we can see the peak before and the peak after has subsequential changes. So, the after peak has the much higher wavelength or higher absorbance than the only chitosan nanoparticle which indicates the successful coating of the drug on the nanoparticles.

❖ Nano coating of the sample (Limonene)

- Although it is a oil sample so directly it is not mixed with chitosan.
- So we take 5.4ml limonene sample then mixed with 20ml distilled water
- Then, let the solution on magnetic sterile for 1 hour.
- Later 40ml HCL added drop wise in the solution.
- Now the chitosan added 15ml dropwise in the solution.
- Then heated the sample for 15 minutes at 50 degree C.
- Now, Filtered the sample to let the oil to float on the surface.
- Let the solution be on magnetic stirrer for 1hr.
- Now we have to maintain the pH of the solution .Ideal pHis 4.8.
- So,first after measuring thr pH of the solution ,it came around pH =1.

- For increasing the pH we used NaOH pallet . after give 6 NaOh pallet the pH is getting increase and get the pH 4.8.
- By converting the solution into salt we get the ideal pH

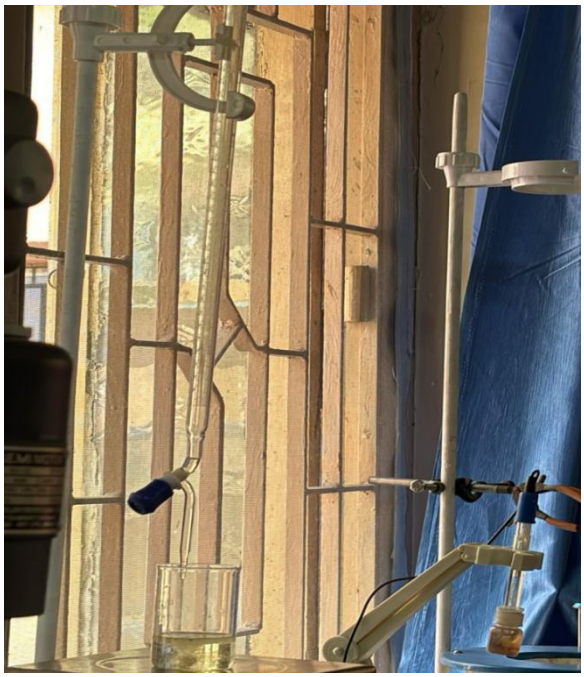

Fig 9: HCL added drop wise

Fig 10: Chitosan added dropwise

Fig 11: Filtered the sample to let the oil to float on the surface

Fig 12 : Maintain the pH using pH meter

❖ Poly Microbial Set Preparation

Historically, evidence for an infectious disease causative agent was obtained by fulfilling Koch's postulates, which require the isolation of the microbe responsible for infection from the site of infection, cultivation in a laboratory, and reproduction of the initial infection by introducing pathogen back into the host. They support the identification of microbial species present at the moment of infection by excluding the presence of other microorganisms in the course of the disease. The theories are based on the assumption that secondary microbial species with low abundances play no role in inciting the disease. However, it is becoming most obvious, even to the most credulous, that most, if not all, diseases are not monomicrobial in nature and manifestation.

Specifically, at this point we believe that many infections initiate—as remain—polymicrobial, similar to most wound infections and CAUTI, or they initiate as polymicrobial inoculation into the infection site.

Indeed, since it was recognized for some time, the role that polymicrobial bacterial communities can play in driving specific disease states—such as otitis media and periodontitis—is relatively well characterized. However, there is emerging consensus that some traditionally considered monomicrobial diseases, such as urinary tract infections, are also driven by other organisms present at lower titers during active infection and by the microbial consortium at the time of infection. The formation of biofilms-often protected environments in which microbial organisms can replicate-is another common feature in chronic and/or recurrent infections. For polymicrobial culture, three or four types of bacteria able to metabolize on their own on one culture are needed with no certain hurdles required. Whereas most bacteria in nature either coexist peacefully with other bacteria or are thoroughly inhibited by them due to a certain bacteriotoxin, in the laboratory environment one unique bacterium is isolated, cultivated and then introduced back with the rest. The primary purpose of testing for antibiotic activity in polymicrobial cultures is to find evidence of broad-spectrum antibiotic action of a synthesised component or phytochemical. While a certain bacteria will cause a different disease depending on the source of contamination and whether the bacterium exists in multicolonial form, it must be able to be infectious at that number. But the goal of exploiting that component in the future is to become an antibiotic to a number of bacteria, meaning that if a drug is made with it, its effectiveness must count to be cost-effective and help the economy of medicines.

Polymicrobial Cohort Preparation

In order to make polymicrobial cultures, 2 different sets of microorganisms were made. The 2 different sets were as follows:

Set 1: Enterococcus faecalis, Listeria monocytogenes, Serratia marcescens

Set 2: Pseudomonas aeruginosa, Campylobacter jejuni, Listeria monocytogenes

Set 1 was made on the basis that both the Enterococcus and Listeria species secretes cytolysin toxin that form pores on the cell membrane of Gram-positive bacteria like Staphylococcus aureus and Lactococcus lactis. Serratia species was made a part of this set as it secretes ShlA toxin that inhibits Gram negative bacteria.

Set 2 was made on the basis that Campylobacter and Listeria survive together in livestock and are the causal agents of several diseases in animals. Pseudomonas forms biofilm in case of environmental stress conditions. Formation of biofilm helped the other two microorganisms along with Pseudomonas to survive together

Microbial colonies from plates of 2 different bacteria for each set was taken using an inoculation loop and then inoculated into liquid LB media of 60 ml and then kept in an incubator at 37°C for 24 hours. After the incubation period, those cultures were again spread on specific media of those three bacteria and the microbes were allowed to grow at 37°C. Next day, each bacterial plate contained 95% of the specific bacteria which confirmed the growth of the three microbes in the poly microbial cohort. Also, staining confirmed the presence of the three microbes in the cohort. The cohort was prepared according to the survivability of the microbes with each other and hence we checked the survivability according the gram character, toxins released and rate of competitive inhibition.

❖ MIC (minimum inhibitory concentration)

The MIC convention decides the most reduced concentration of an antimicrobial specialist required to hinder the unmistakable development of a microorganism in a controlled environment. It's a standard strategy utilized in antimicrobial helplessness testing. MIC testing includes planning a extend of anti-microbial concentrations and uncovering them to a standardized bacterial inoculum, regularly in broth or agar.

The Steps Of Doing MIC As Follows

1. Bacterial Plate Preparation:

i. Media of 45ml (with agar) was prepared and heated
ii. Then wait for sometime so that it cool down properly.
iii. Later we pour them into 5 different plates
iv. Added 5 different types of bacterial culture into 5 different types of plate
v. Spreading was done
vi. Seal the plate using tape
vii. Then,we kept the plates in incubator for 24hours for the growth of bacteria.

2. Inoculation:

i. Firstly we make LB media and inoculate poly set of bacteria for 2set.
ii. Then,kept the media in shaker.
iii. After some hours we cheack the OD in spectrophotometer and wait until the OD reached 0.58 to 0.61.
iv. Then 3ml media kept diffently for standard solution and then added the drug sample(limonene) for 2 different poly set. (By calculating in this V1 = (C2 * V2) / C1formula we added our drug in the media)
v. The we checked 3 different OD of standard and dosage in 1hour of interval.

❖ BACTERIAL

Two types of polyset bacteria are used of mic (minimum inhibitory concentration)

1) **Set1 MIC**

➢ Name of bacteria used in polyset 1 MIC
Enterococcus faecalis, Listeria monocytogenes, Serratia marcescens

C1 = 56325 µg/ml (initial concentration)
V2 = 3 ml (final volume)
C2 = 500µg/ml (final concentration)
V1 = ?(Drug Volume)
V1 = (C2 * V2) / C1 = (500 * 3) / 56325 ≈ 0.0266 ml or 26.6µl, which is approximated to 27µl.
Starting OD =0.61

2) **Set2 MIC**

Name of bacteria used in polyset 2 MIC
Pseudomonas aeruginosa, Campylobacter jejuni, Listeria monocytogenes

C1 = 56325 µg/ml (initial concentration)
V2 = 3 ml (final volume)
C2 = 500µg/ml (final concentration)
V1 = ?(Drug Volume)
V1 = (C2 * V2) / C1 = (500 * 3) / 56325 ≈ 0.0266 ml or 26.6µl, which is approximated to 27µl.

❖ IC50(Half-maximal inhibitory concentration)

IC50 refers to the concentration of a compound that's required to decrease a organic movement by half.

The "half" shows that the impact is decreased to 50% of its unique level when the substance is display at the IC50 concentration.

Like MIC here also we used 2 poly set and mesured the OD of standard and dosage same like MIC.

For IC50 ,same polyset of bacteria have been used that have been used for MIC set 1.

C1 = 56325 µg/ml (initial concentration)
V2 = 3 ml (final volume)
C2 = 600µg/ml (final concentration)
V1 = ?(Drug Volume)
V1 = (C2 * V2) / C1 = (600 * 3) / 56325 ≈
0.03195 ml or 31.957 µl, which is approximated to 32 µl.

- For IC50 ,same polyset of bacteria have been used that have been used for MIC set 2

C1 = 56325 µg/ml (initial concentration)
V2 = 3 ml (final volume)
C2 = 715µg/ml (final concentration)
V1 = ?(Drug Volume)
V1 = (C2 * V2) / C1 = (715 * 3) / 56325 ≈
0.03808 ml or 38.08 µl, which is approximated to 38 µl.

❖ ZOI(Zone Of Inhibition):

Zone Of Inhibition is a clear zone around an antimicrobial agent on a growth medium where microbial development is repressed.This region demonstrates the adequacy of the antimicrobial against the microbes
We have done ZOI by using polyset 1 MIC bacteria .

Enterococcus faecalis, Listeria monocytogenes, Serratia marcescens

HOW IT WORK

- First we prepared LB agar media and heated .
- Then poured the agar media into a petri dish.
- Then wait for sometime so that it cool down properly.
- Then we make a well in the middle of the plate and add polyset bacteria culture approx 100µl.
- we kept the plates in incubator for 24hours
- Next day we saw the ZOI in the plate
- Then the diameter of the ZOI was measured

❖ FUNGI

FUNGAL MIC

The fungi we used to see MIC is *Aspergillus niger*.

C1 = 56325 µg/ml (initial concentration)
V2 = 3 ml (final volume)
C2 = 500µg/ml (final concentration)
V1 = ?(Drug Volume)
V1 = (C2 * V2) / C1 = (500 * 3) / 56325 ≈ 0.0266 ml or 26.6µl, which is approximated to 27µl.
Starting OD =0.61

FUNGAL IC50

C1 = 56325 µg/ml (initial concentration)
V2 = 3 ml (final volume)
C2 = 715µg/ml (final concentration)
V1 = ?(Drug Volume)
V1 = (C2 * V2) / C1 = (715 * 3) / 56325 ≈
0.03808 ml or 38.08 µl, which is approximated to 38 µl.
Starting OD =0.58

❖ ZOI(Zone Of Inhibition)

Zone Of Inhibition is a clear zone around an antimicrobial agent on a growth medium where microbial development is repressed.This region demonstrates the adequacy of the antimicrobial against the microbes.

We have done ZOI by using fungi. The fungi we used to see the ZOI is *Aspergillus niger.*

HOW IT WORK

- First we prepared PDA agar media and heated .
- Then poured the agar media into a petri dish.
- Then wait for sometime so that it cool down properly.
- Then we make a well in the middle of the plate and add the fungal culture approx 100µl.
- we kept the plates in incubator for 24hours
- Next day we saw the ZOI in the plate
- Then the diameter of the ZOI was measured

RESULT AND DISCUSSIONS

✧ The data obtained from set 1 MIC

Starting OD =0.61

Table number 1(Result of polyset 1 bacterial MIC)

Standard	Dosage
0.62	0.60
0.64	0.61
0.79	0.64

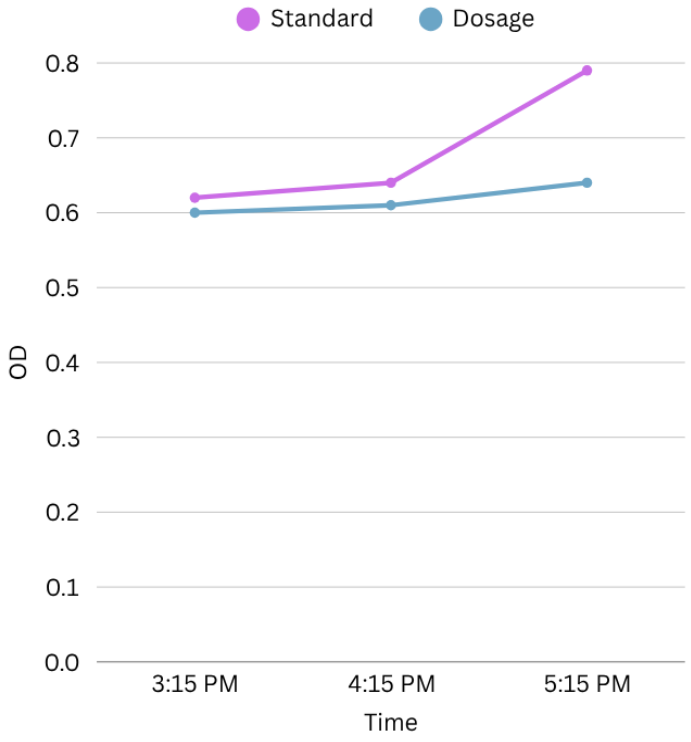

Fig13: Result of Set1 MIC

✧ The data obtained from set 2 MIC

Starting OD =0.61

Table number 2(Result of polyset 2 bacterial MIC)

Standard	Dosage
0.72	0.61
0.76	0.62
0.80	0.64

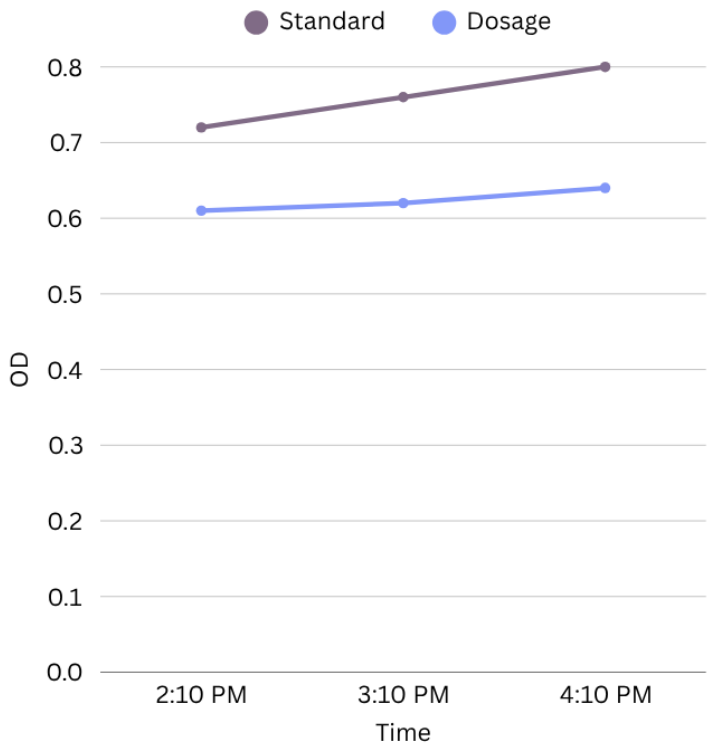

Fig14: Result of Set2 MI

✧ The data obtained from set 1 IC50

Starting OD =0.58

Table number 3(Result of polyset 2 bacterial IC50)

Standard	Dosage
0.50	0.48
0.50	0.43
0.45	0.36

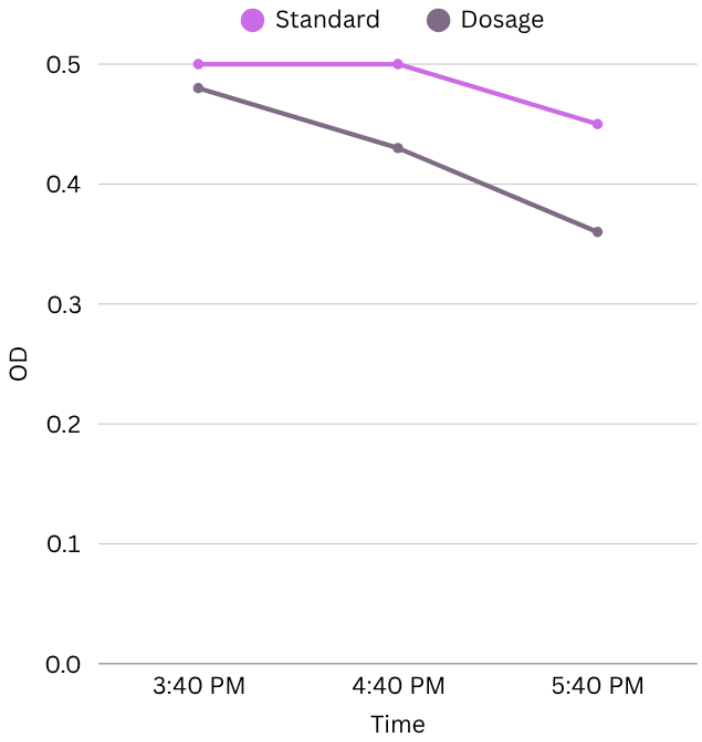

Fig15: Result of Set1 IC50

✧ The data obtained from set 2 IC50

Starting OD =0.58

Table number 4(Result of polyset 2 bacterial IC50)

Standard	Dosage
0.53	0.47
0.55	0.42
0.50	0.37

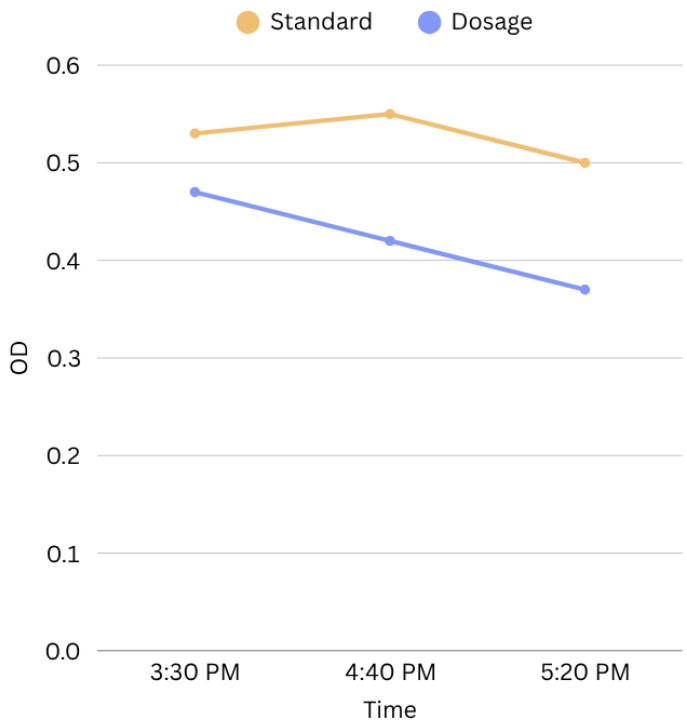

Fig16: Result of Set 2 IC50

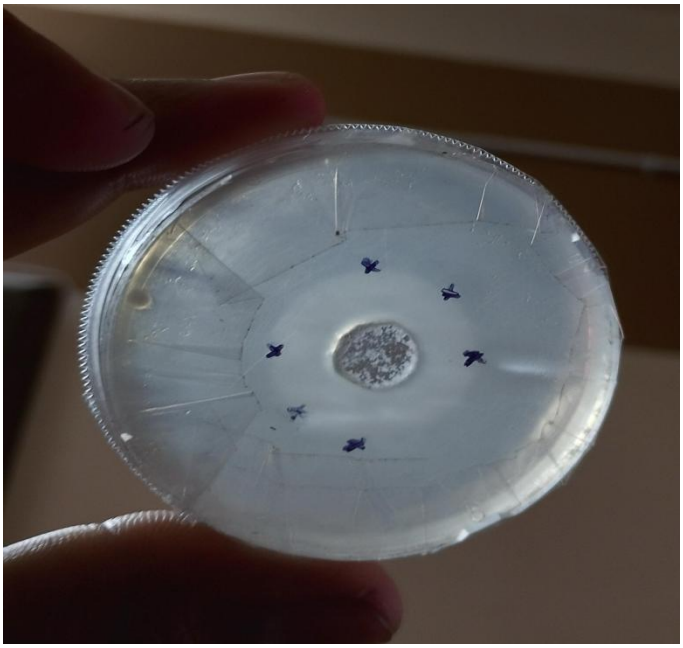

Fig17: A clear Zone Of Inhibition is observed in the figure (Bacteria)

✧ The data obtained from MIC

The fungi we used to see MIC is *Aspergillus niger*.

Starting OD =0.61

Table number 5(The result of fungal MIC)

Standard	Dosage
0.66	0.60
0.71	0.61
0.79	0.65

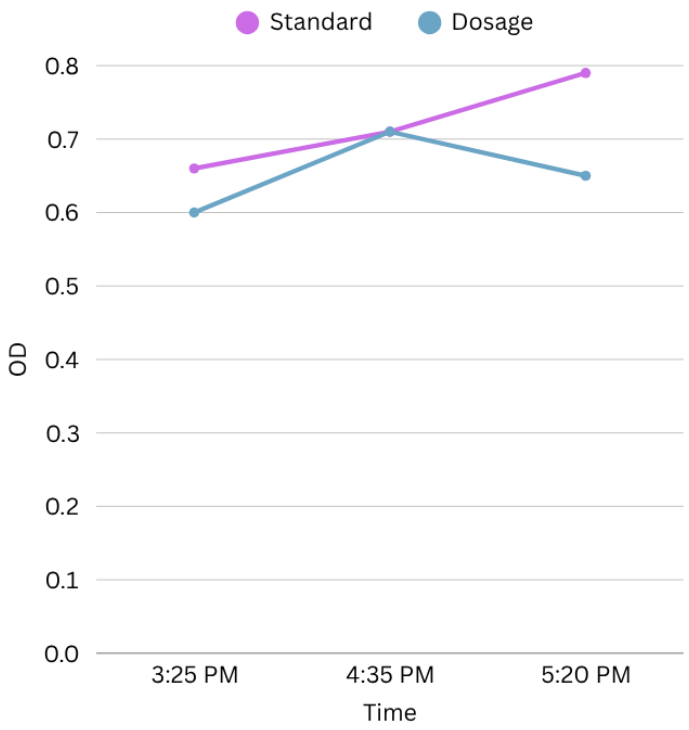

Fig18: Result of MIC

✧ The data obtained from IC50

Starting OD =0.58

Table number 6 (The result of fungal IC50)

Standard	**Dosage**
0.56	0.48
0.55	0.42
0.48	0.36

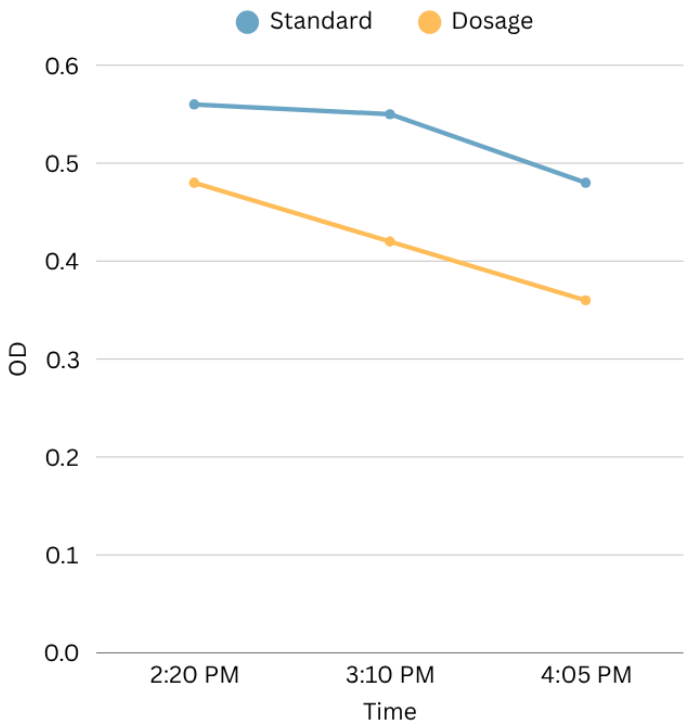

Fig19: Result of IC50

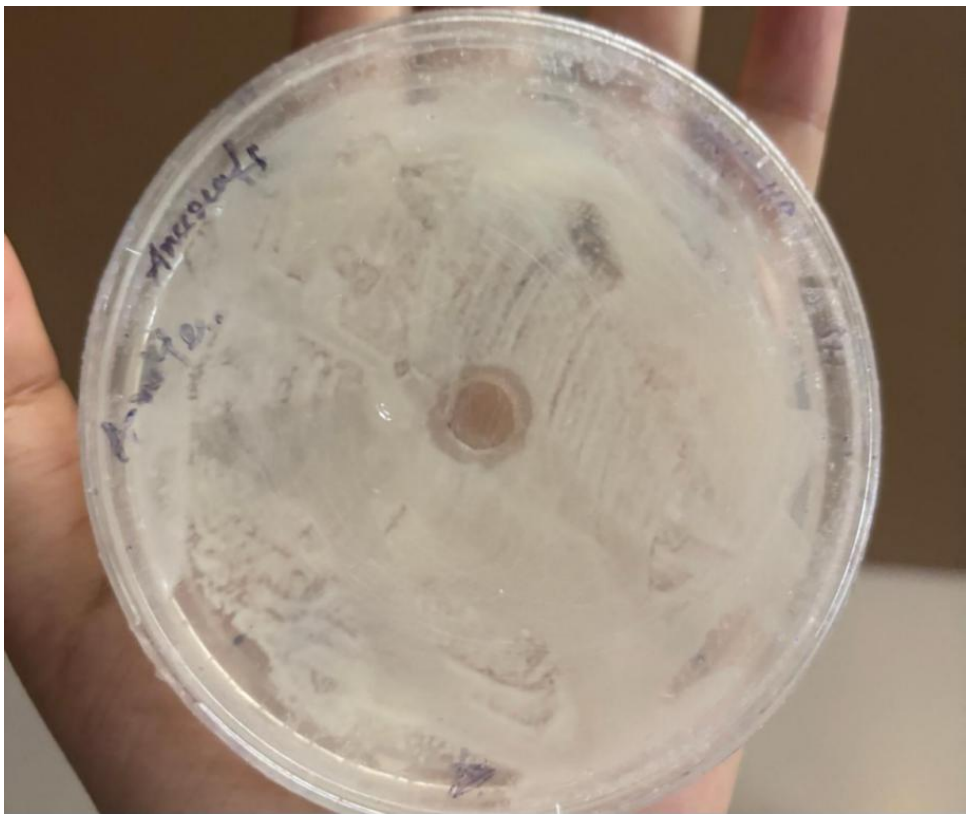

Fig20: A clear Zone Of Inhibition is observed in the figure(Fungi)

CONCLUSION

After carried out all of the experiments from the extraction of limonene from sweet lime,we can easily conclude that the extracted limonene has antimicrobial property. These finding proves that use of limonene as natural antimicrobial agent,which could be beneficial for food preservation,natural cleaning products or pharmacutical.

APPENDIX

1. Introduction

2. Review of Literature

3. Materials and methods

- **Extraction, Isolation and Purification of Secondary Metabolites**
- **Biochemical test**
- **TLC(Thin Layer Chromatography)**
- **UV spectrophotometer analysis**
- **Nano particle preparation of chitosan**
- **Nano coating of the sample(Limonene)**
- **Poly Microbial Set Preparation**
- **MIC(minimum inhibitory concentration)**
- **ZOI(Zone Of Inhibition)**

4. RESULT AND DISCUSSIONS

5.CONCLUSION

REFERENCE

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DEVELOPMENT OF SECONDARY METABOLITES CONJUGATED WITH NANO POLYMERS AGAINST POLY MICROBIAL INFECTIONS

A PROJECT SUBMITTED BY

Anurati Mandal
Enrollment ID : 2322211001016

Under the Supervision of
Mr Mayukh Bose
Bose Informatics

53/F, Feeder Road, Beehive Garden,
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Certificate From Current Institute

DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled "DEVELOPMENT OF SECONDARY METABOLITES CONJUGATED WITH NANO POLYMERS AGAINST POLY MICROBIAL INFECTIONS IN CULTUR" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Microbiology is an authentic record of my own work carried out during the period from February 2025 to May 2025 under the supervision of Mr Mayuk Bose Department of Biotechnology. I acknowledge that I have read and understood the UGC regulations, 2018 (Promotion of Academic integrity and Prevention of Plagiarism in Higher Educational Institutions).

I confirm that this dissertation has been checked for plagiarism using the plagiarism checking software----------. At the end of the dissertation, a copy of the summary report demonstrating similarities in content and its potential source (if any), generated online using plagiarism checking software is enclosed. I herewith confirm that the dissertation has been less than 10% similarity according to the plagiarism checking software's report and meets the UGC Regulations as well as plagiarism rules of Sister Nivedita University.

I further declare that no portion of the dissertation or its data will be published without the Institute's or supervisor's permission. I have not previously applied for any other degree or award using the topics and findings described in my dissertations.

(Signature of the students with Date)

Name of the Student: Anurati Mandal
Registration no: 230010161830
Department: Microbiology

M.sc Ceirtificate
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Abbreviations

- TLC :Thin Layer Chromatography
- TPP: Sodium tripolyphosphate
- MIC:Minimum inhibitory concentration
- ZOI:Zone Of Inhibition

ABSTRACT

Limonene, a naturally occurring monocyclic monoterpene, is likely one of the principle constituents of citronaceous osnovoy oils and possesses remarkable industrial importance due to its use in food flavoring, cosmetics, medicine, and biopertroleum solvents. This research work focuses on the retrieval, purification and partitioning of limonene from sweet lime (Citrus limetta) waste peels which are normally regarded as waste. The main aim is to develop a recovery technique that is environment-friendly and economically viable and enhances the value of agricultural wastes.After Extraction, Isolation and Purification of the sample ,we did nano particle preparation of chitosan.Then we nano coated our sample. although it is a oil sample so directly it is not mixed with chitosan.So first we take 5.4ml limonene sample then mixed with 20ml distilled water.Then,let the solution on magnetic sterile for 1 hour.Later 40ml HCL added drop wise in the solution. Now the chitosan added

15ml dropwise in the solution.Then heated the sample for 15 minutes at 50 degree C.Now,Filtered the sample to let the oil to float on the surface.Let the solution be on magnetic stirrer for 1hr.Now we have to maintain the ph of the solution .Ideal pH is

4.8.So,first after measuring the pH of the solution ,it came around pH=1.For increasing the PH we used NaOH pallet . after give 6 NaOh pallet the pH is getting increase and get the pH 4.8.By converting the solution into salt we get the ideal pH. Then we performed MIC of 2 poly microbial sets and done IC50 with the same polymicrobial set.And fungal MIC and IC50 we did using a strain of fungi. Then, we did ZOI both bacteria and fungi.After carried out all of the experiments from the extraction of limonene from sweet lime,we can easily conclude that the extracted limonene has antimicrobial property.

Keywords

Limonene ,Sweetlime, Enterococcus faecalish,
Listeria monocytogenes,TLC,Zone of inhibition

Contents

□ Introduction
□ Review of Literature
□ Materials and methods
□ Results and discussions
□ Conclusion
□ Appendix
□ References
□ Plagiarism report

INTRODUCTION Limonene, found in citrus oils, such as lemons and oranges, has a monocyclic structure with a chemical makeup of $C_{10}H_{16}$. Citrus fruits contain rinds that predominate its existence in the form of D-isomer, which gives lemongrass its distinctive flavor. It is being actively pursued in the industry and various scientific fields owing to its economic significance and versatility in agriculture, pharmaceuticals, green chemistry, and flavor industries. Also, because of its anti-inflammatory and potential anti-cancer properties, limonene is being explored as a therapeutic agent, diabolic cleanser, natural solvent of greener origins and an environmentally-friendly cleaner.Eco-friendly extraction practices include purification, extraction, and the processes of limonene isolation. The triadic synergetic system forms the provincial axis of accomplishing optim maximization of the results. Coping with myriade variants and multileveled tasks entails to layer the extracted system using a special space approach while systematically remaining in the bounds of provisional means.Enhanced technology deals with multiplicity of selection of streams like refined air scrubbing, concentrated watery solution, steam distillation, and solvent distillation. Instead of harmful organic solvents which need to be disposed of, separating the compound via steam distillation becomes very beneficial and convenient for laboratories and industries because of its uncomplicated approach, ultimate effectiveness and commonly known steam sifting methods and practice.Limonene exerts marked inhibitory activity against different pathogens, such as with Listeria monocytogenes, a bacterium that challenges food safety. It binds to the cell membrane and interrupts its structure, which causes the membrane to become permeable and leakage of its cytosol. Furthermore, limonene blocks bacterial energy metabolism by disrupting the respiratory chain and ATPase, which disables the synthesis of ATP. In addition, it has been documented that limonene can lessen the severity of Pseudomonas aeruginosa infection by blocking quorum sensing. The Pseudomonas aeruginosa biofilm accounts for 80% of the total biofilm formed. This leads to decreased biofilm development and the bioinduced virulence factors elastase and pyocyanin.Also, limonene proved to exhibit vigorous antifungal activity. Trichophyton rubrum, one of the dermatophytes that cause onychomycosis, was inhibited in vitro. At a concentration of 0.5% v/v, limonene demonstrates this effect with C at this concentration. As well, it limits the growth and virulence of highly pathogenic yeast known as Candida parapsilosis by blocking sculpting.

Review of literature

Limonene, a naturally occurring monocyclic monoterpene, is likely one of the principle constituents of citronaceous osnovoy oils and possesses remarkable industrial importance due to its use in food flavoring, cosmetics, medicine, and biopertroleum solvents. This research work focuses on the retrieval, purification and partitioning of limonene from sweet lime (Citrus limetta) waste peels which are normally regarded as waste. The main aim is to develop a recovery technique that is environment-friendly and economically viable and enhances the value of agricultural wastes.Limonene, found in citrus oils, such as lemons and oranges, has a monocyclic structure with a chemical makeup of $C_{10}H_{16}$. Citrus fruits contain rinds that predominate its existenceD-isomer, which gives lemongrass its distinctive flavor. It is being actively 4 in the form of pursued in the industry and various scientific fields owing to its economic significance and versatility in 5 because of its anti-inflammatory agriculture, pharmaceuticals, green chemistry, and flavor industries. Also, and potential anti-cancer properties, limonene is being explored as a therapeutic agent, diabolic cleanser, natural solvent of greener origins and an environmentally-friendly cleaner.Eco-friendly extraction practices include purification, extraction, and the processes of limonene isolation. Enhanced technology deals with multiplicity of selection of streams like refined air scrubbing, concentrated watery solution, steam distillation, and solvent distillation. Instead of harmful organic solvents which need to be disposed of, separating the compound via steam distillation becomes very beneficial and convenient for laboratories and industries because of its uncomplicated approach, ultimate effectiveness and commonly known steam sifting methods and practice.Limonene exerts marked inhibitory activity against different pathogens, such as with Listeria monocytogenes, a bacterium that challenges food safety. It binds to the cell membrane and interrupts its structure, which causes the membrane to become permeable and leakage of its cytosol. Furthermore, limonene blocks bacterial energy metabolism by disrupting the respiratory chain and ATPase, which disables the synthesis of ATP. In addition, it has been documented that limonene can lessen the severity of Pseudomonas aeruginosa infection by blocking quorum sensing. The Pseudomonas aeruginosa biofilm accounts for 80% of the total biofilm formed. This leads to decreased biofilm development and the bioinduced virulence factors elastase and pyocyanin.Also, limonene proved to exhibit vigorous

antifungal activity. Trichophyton rubrum, one of the dermatophytes that cause onychomycosis, was inhibited in vitro. At a concentration of 0.5% v/v, limonene demonstrates this effect with C at this concentration. As well, it limits the growth and virulence of highly pathogenic yeast known as Candida parapsilosis by blocking sculpting.

Materials and Methods

 Extraction, Isolation and Purification of Secondary Metabolites:
Graded sweet lime peels from approximately 23 sweet lime are taken.

I. Extraction

1.The graded peels are transferred to a round bottom flask.
2.To the peels, 400 ml of distilled water is poured.
3.A simple water bath distillation is carried out.
4.Two separate regions will form in the distillate portion, the upper portion making limonene.

II. Isolation and purification

The whole distillateto 7 is transferred to a separatory funnel separate out the upper limonene layer.

 Biochemical test

2ml of chloroform is taken and mixed with 0.5 ml of sample. Conc H_2SO_4 added drop wise. Then, ring of red brown colour forms monoterpenoid present and confirmed.

 TLC(Thin Layer Chromatography)
Silica is powdered using mortar pestle and distilled water is added then dried into the air
oven forming a thin layer. In TLCexplain the distance between the sample and the solvent. So Rf factor is basically defined as the sample divided by distance travelled by the solvent.

6 ,Rf value or Retention Factor

the distance travelled by

For TLC Chloroform 16ml and Hexon 4ml mixed like 8:2 ratio.

Rf value =Sample distance/solvent distance
1.7/5.4 = .315
The RF value given in protocol is .2 to.3
And,the RF I got is .3.So the purity is maintained by doing TLC.
 UV spectrophotometer analysis
Maintain the OD at 237 nm, after that the uv spectra result is 0.023 this result is same that given in the protocol.

 Nano particle preparation of chitosan
 50 mg of chitosan powder dissolved in 50mL deionized distilled water.

 After in at room temperature, the solution placed on the magnetic stirring and 1% acetic acid solution added dropwise within 30 mins to create the homogeneous. chitosan solution.

 Sodium tripolyphosphate [TPP 0.5% (W/V), 2mL] as a crosslinking agent added drop wise and the compound will be stirred 30 meus.

 2.0 mg/mL ICG added to the prepared chitosan solution and mixed via a magnetic mixer for 10 meus at 1000 rpm, în at pH 4.8.

 Eventually, this solution centrifuged at 10,000 ppm for 30mins.
 The entire chitosan nanoparticle solution is passed through a 0.22 micron syringe filter to make sure particles below the range of 200 nm passes through the solution after which the entire solution is scanned from 200-800 nm in spectrophotometer which should give a peak concluding nanoparticles are formed.

Fig:The UV range in spectrophotometer without the coating of the drug
 Once the nanoparticles are coated with drugs again the solution is scanned through a UV Spectrophotometer from 200-800 nm and again the peak is observed.

Now in comparision we can see the peak before and the peak after has subsequential changes. So, the after peak has the much higher wavelength or higher absorbance than the only chitosan nanoparticle which indicates the successful coating of the drug on the nanoparticles.

 Nano coating of the sample(Limonine)

 Although it is a oil sample so directly it is not mixed with chitosan. So first we take 5.4ml limonine sample then mixed with 20ml distilled water

 Then,let the solution on magnetic sterile for 1 hour.
 Later 40ml HCL added drop wise in the solution.
 Now the chitosan added 15ml dropwise in the solution.
 Then heated the sample for 15 minutes at 50 degree C.
 Now,Filtered the sample to let the oil to float on the surface.
 Let the solution be on magnetic stirrer for 1hr.
 Now we have to maintain the pH of the solution .Ideal pHis 4.8.
 So,first after measuring thr pH of the solution ,it came around pH =1.
 For increasing the pH we used NaOH pallet . after give 6 NaOh pallet the pH is getting increase and get the pH 4.8.

 By converting the solution into salt we get the ideal pH
 Poly Microbial Set Preparation
Historically, evidence for an infectious disease causative agent was obtained by fulfilling Koch's postulates,

which require the isolation of the microbe responsible for infection from the site of infection, cultivation in a laboratory, and reproduction of the initial infection by introducing pathogen back into the host. They support the identification of microbial species present at the moment of infection by excluding the presence of other microorganisms in the course of the disease. The theories are based on the assumption that secondary microbial species with low abundances play no role in inciting the disease. However, it is becoming most obvious, even to the most credulous, that most, if not all, diseases are not monomicrobial in nature and manifestation. Specifically, at this point we believe that many infections initiate—as remain—polymicrobial, similar to most wound infections and CAUTI, or they initiate as

polymicrobial inoculation into the infection site.

Indeed, since it was recognized for some time, the role that polymicrobial bacterial communities can play in driving specific disease states—such as otitis media and periodontitis—is relatively well characterized. However, there is emerging consensus that some traditionally considered monomicrobial diseases, such as urinary tract infections, are also driven by other organisms present at lower titers during active infection and by the microbial consortium at the time of infection. The formation of biofilms-often protected environments in which microbial organisms can replicate-is another common feature in chronic and/or recurrent infections. For polymicrobial culture, three or four types of bacteria able to metabolize on their own on one culture are needed with no certain hurdles required. Whereas most bacteria in nature either coexist peacefully with other bacteria or are thoroughly inhibited by them due to a certain bacteriotoxin, in the laboratory environment one unique bacterium is isolated, cultivated and then introduced back with the rest. The primary purpose of testing for antibiotic activity in polymicrobial cultures is to find evidence of broad-spectrum antibiotic action of a synthesised component or phytochemical. While a certain bacteria will cause a different disease depending on the source of contamination and whether the bacterium exists in multicolonial form, it must be able to be infectious at that number. But the goal of exploiting that component in the future is to become an antibioticbacteria, meaning that if a drug is made with it, its

effectiveness must count to be cost-effective and help the economy of medicines.

5 to a number of

Polymicrobial Cohort Preparation

In order to make polymicrobial cultures, 2 different sets of microorganisms were made. The 3 different sets were as follows:

Set 1: Enterococcus faecalis, Listeria monocytogenes, Serratia marcescens
Set 2: Pseudomonas aeruginosa, Campylobacter jejuni, Listeria monocytogenes

Set 1 was made on the basis that both the Enterococcus and Listeria species secretes cytolysin toxin that form pores on the cell membrane of Gram-positive bacteria like Staphylococcus aureus and Lactococcus lactis. Serratia species was made a part of this set as it secretes ShlA toxin that inhibits Gram negative bacteria.

Set 2 was made on the basis that Campylobacter and Listeria survive together in livestock and are the causal agents of several diseases in animals. Pseudomonas forms biofilm in case of environmental stress conditions. Formation of biofilm helped the other two microorganisms along with Pseudomonas to survive together

Microbial colonies from plates of 2 different bacteria for each set was taken using an inoculation loop and then inoculated into liquid LB media of 60 ml and then kept in an incubator at 37°C for 24 hours. After the incubation period, those cultures were again spread on specific media of those three bacteria and the microbes were allowed to grow at 37°C. Next day, each bacterial plate contained 95% of the specific bacteria which confirmed the growth of the three microbes in the poly microbial cohort. Also, staining confirmedthree microbes in the cohort. The cohort was prepared according to 1 the presence of the the survivability of the microbes with each other and hence we checked the survivability according the gram character, toxins released and rate of competitive inhibition.

 MIC(minimum inhibitory concentration)
The MIC convention decides the most reduced concentration of an antimicrobial specialist required to hinder the unmistakable development of a microorganism in a controlled environment. It's a standard strategy utilized in antimicrobial helplessness testing. MIC testing includes planning a extend of anti-microbial concentrations and uncovering them to a standardized bacterial inoculum, regularly in broth or agar.

The Steps Of Doing MIC As Follows

1. Bacterial Plate Preparation:
i. Media of 45ml (with agar) was prepared and heated ii. Then wait for sometime so that it cool down properly. iii. Later we pour them into 5 different plates iv. Added 5 different types of bacterial culture into 5 different types of plate

v. Spreading was done vi. Seal the plate using tape vii. Then,we kept the plates in incubator for 24hours for the growth of bacteria.

2. Inoculation:
i. Firstly we make LB media and inoculate poly set of bacteria for 2set.

ii. Then,kept the media in shaker.

iii. After some hours we cheack the OD in spectrophotometer and wait until the OD reached 0.58 to 0.61.

iv. Then 3ml media kept diffently for standard solution and then added the drug sample(limonene) for 2 different poly set. (By calculating in this V1 = (C2 * V2) / C1formula we added our drug in the media)

v. The we checked 3 different OD of standard and dosage in 1hour of interval.

 BACTERIAL

Two types of polyset bacteria are used of mic (minimum inhibitory concentration)
1) Set1 MIC

 Name of bacteria used in polyset 1 MIC
Enterococcus faecalis, Listeria monocytogenes, Serratia marcescens

C1 = 56325 µg/ml (initial concentration)
V2 = 3 ml (final volume)
C2 = 500µg/ml (final concentration)
V1 = ?(Drug Volume)
V1 = (C2 * V2) / C1 = (500 * 3) / 56325 ≈ 0.0266 ml or 26.6µl, which is approximated to 27µl.

Starting OD =0.61

2) Set2 MIC

Name of bacteria used in polyset 2 MIC
Pseudomonas aeruginosa, Campylobacter jejuni, Listeria monocytogenes

C1 = 56325 µg/ml (initial concentration)
V2 = 3 ml (final volume)
C2 = 500µg/ml (final concentration)
V1 = ?(Drug Volume)
V1 = (C2 * V2) / C1 = (500 * 3) / 56325 ≈ 0.0266 ml or 26.6µl, which is approximated to 27µl.

 IC50(Half-maximal inhibitory concentration)

IC50 refers to the concentration of a compound that's required to decrease a organic movement by half.

The "half" shows that the impact is decreased to 50% of its unique level when the substance is display at the

IC50 concentration.

Like MIC here also we used 2 poly set and mesured the OD of standard and dosage same like MIC.

For IC50 ,same polyset of bacteria have been used that have been used for MIC set 1.
C1 = 56325 µg/ml (initial concentration)
V2 = 3 ml (final volume)
C2 = 600µg/ml (final concentration)
V1 = ?(Drug Volume)
V1 = (C2 * V2) / C1 = (600 * 3) / 56325 ≈
0.03195 ml or 31.957 µl, which is approximated to 32 µl.

 For IC50 ,same polyset of bacteria have been used that have been used for MIC set 2
C1 = 56325 µg/ml (initial concentration)
V2 = 3 ml (final volume)
C2 = 715µg/ml (final concentration)
V1 = ?(Drug Volume)
V1 = (C2 * V2) / C1 = (715 * 3) / 56325 ≈
0.03808 ml or 38.08 µl, which is approximated to 38 µl.

1 Zone Of Inhibition is a clear zone around an antimicrobial agent ZOI(Zone Of Inhibition): on a growth medium

where microbial development is repressed.This region demonstrates the adequacy of the antimicrobial against

the microbes

We have done ZOI by using polyset 1 MIC bacteria .
Enterococcus faecalis, Listeria monocytogenes, Serratia marcescens
How it work

 First we prepared LB agar media and heated .
 Then poured the agar media into a petri dish.
 Then wait for sometime so that it cool down properly.

 Then we make a well in the middle of the plate and add polyset bacteria culture approx

100µl.
 we kept the plates in incubator for 24hours
 Next day we saw the ZOI in the plate
 1 the diameter of the ThenZOI was measured

 FUNGI

FUNGAL MIC
The fungi we used to see MIC is Aspergillus niger.

C1 = 56325 µg/ml (initial concentration)
V2 = 3 ml (final volume)
C2 = 500µg/ml (final concentration)
V1 = ?(Drug Volume)
V1 = (C2 * V2) / C1 = (500 * 3) / 56325 ≈ 0.0266 ml or 26.6µl, which is approximated to 27µl.
Starting OD =0.61

FUNGAL IC50

C1 = 56325 µg/ml (initial concentration)
V2 = 3 ml (final volume)
C2 = 715µg/ml (final concentration)
V1 = ?(Drug Volume)
V1 = (C2 * V2) / C1 = (715 * 3) / 56325 ≈
0.03808 ml or 38.08 µl, which is approximated to 38 µl.
Starting OD =0.58

 ZOI(Zone Of Inhibition)

1 Zone Of Inhibition is a clear zone around an antimicrobial agent on a growth medium where microbial development is repressed.This region demonstrates the adequacy of the antimicrobial against the microbes.

We have done ZOI by using fungi. The fungi we used to see the ZOI is
Aspergillus niger.

How it work

 First we prepared PDA agar media and heated .
 Then poured the agar media into a petri dish.
 Then wait for sometime so that it cool down properly.

 Then we make a well in the middle of the plate and add the fungal culture approx 100µl. we kept the plates in incubator for 24hours

 Next the diameter of day we saw the ZOI in the plate

 1 the ThenZOI was measured

.

RESULT AND DISCUSSIONS

 The data obtained from set 1 MIC

Starting OD =0.61

Table number 1(Result of polyset 1 bacterial MIC) Standard

Dosage

0.62

0.60

0.64

0.61

0.79

0.64

Fig:Result of Set1 MIC

 The data obtained from set 2 MIC

Starting OD =0.61

Table number 2(Result of polyset 2 bacterial MIC) Standard

Dosage

0.72

0.61

0.76

0.62

0.80

0.64

Fig:Result of Set2 MIC

 The data obtained from set 1 IC50

Starting OD =0.58

Table number 3(Result of polyset 2 bacterial IC50)

Standard

Dosage

0.50

0.48

0.50

0.43

0.45

0.36

Fig: Result of Set1 IC50
 The data obtained from set 2 IC50

Starting OD =0.58

Table number 4(Result of polyset 2 bacterial IC50)

Standard

Dosage

0.53

0.47

0.55

0.42

0.50

0.37

Fig: Result of Set 2 IC50

Fig: A clear Zone Of Inhibition is observed in the figure(Bacteria)

 The data obtained from MIC

The fungi we used to see MIC is Aspergillus niger.

Starting OD =0.61

Table number 5(The result of fungal MIC)

Standard	Dosage
	0.66
	0.60
	0.71
	0.61
	0.79
	0.65

Fig: Result of MIC

 The data obtained from IC50

Starting OD =0.58

Table number 6 (The result of fungal IC50)

Standard

Dosage

0.56

0.48
0.55

0.42

0.48

0.36

Fig: Result of 1 Zone Of Inhibition is IC50 Fig: A clearobserved in the figure(Fungi)

CONCLUSION

After carried out all of the experiments from the extraction of limonene from sweet lime,we can easily conclude that the extracted limonene has antimicrobial property. These finding proves that use of limonene as natural antimicrobial agent,which could be beneficial for food preservation,natural cleaning products or pharmacutical.

Sources

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